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Interaction between Erythrocyte Plasma Membrane and Silicate Dusts

by S. V. Singh,* P. N. Viswanathan* and Q. Rahman†

Lysis of erythrocytes *in vitro* leading to release of hemoglobin has often been used as a convenient test system for predictive toxicological evaluation of noxious particulates encountered as pollutants in occupational and environmental atmospheres. However, in spite of several studies with silica and silicate dusts, the exact biochemical mechanism for membrane damage is not clear. Therefore, detailed biochemical studies were conducted. Screening of a large number of fugitive dust samples indicated that the dissolution of silica under assay conditions has a qualitative relation to hemolytic potency.

The strong capacity of slate mine dust to cause hemolysis was studied in detail. The kinetics of lysis indicated that on prolonged contact the adsorption of hemoglobin altered the lytic index. Coating of dust with serum, albumin or polyvinylpyrrolidone (PVP) and some lipids reduced lytic potency markedly, while EDTA was ineffective. Altering the surface chemistry of dust by heating, alkali treatment, washing with water, and acid washing reduced hemolysis in increasing order. Thus, chemical interactions between dusts and membranes are involved in hemolysis, and it may be concluded that the interaction of dust constituents with biomembranes is one of the likely mechanisms involved in the toxicity of particulate air pollutants.

Introduction

In predictive toxicological evaluation, suitable *in vitro* model systems have a distinct advantage over *in vivo* systems because of their simplicity, because they permit screening a large number of samples simultaneously under comparable conditions, and because of economy of time. Lysis of human erythrocytes in isotonic condition is one such system used for understanding the relative toxicity of different xenobiotics. The experience gained in different laboratories (*1-4*) clearly indicates a positive correlation between hemolytic effect and cytotoxicity of various dusts. The system has been extensively employed in this laboratory to correlate the hemolytic potency of different silicates with their biochemical effects in tissue (*5-8*) and has greatly helped in understanding the biochemical processes underlying the interaction of dusts with biomembranes (*9-12*). The hemolytic potential of a large number of dust samples has been compared under the auspices of a large-scale Environmental Protection Agency (EPA) screening project. Slate dust from Mandsaur, India, had a high hemolytic potency. Also, occupations involving slate dust are known to cause pneumoconiosis (*13*), and the health problems associated with it have attracted the attention in the public press. To the best of our knowledge, there were no experimental reports regarding the cytotoxicity of the Indian variety of slate dust. In the present report, the relation between the solubility of slate dust with both hemolysis and the RBC ghost membrane was explored in physiological media constituents in view of the solubility theory of pathogenesis of pneumoconiosis (*10,11,14,15*). Also, the effects of various pretreatments on the solubility of various components of the slate dust were evaluated.

*Industrial Toxicology Research Centre, Post Box 80, Lucknow 226 001, India.

†National Center for Toxicological Research, Jefferson, AR 72079.

Hemolytic Index of Different Dust Samples

The lysis of a 0.2% suspension of human erythrocytes in 0.01 *M* Tris HCl buffer, pH 7.35, in 0.15 *M* NaCl, caused by different dusts in different amounts, was measured (*10*). The time of incubation was 10 min for chrysotile to avoid adsorption of hemoglobin and 2 hr for other dusts. The concentration (mg/mL) needed for 50% lysis was calculated graphically and expressed as the hemolytic index (HI). The data are given in Figure 1. The Union Internationale Contre

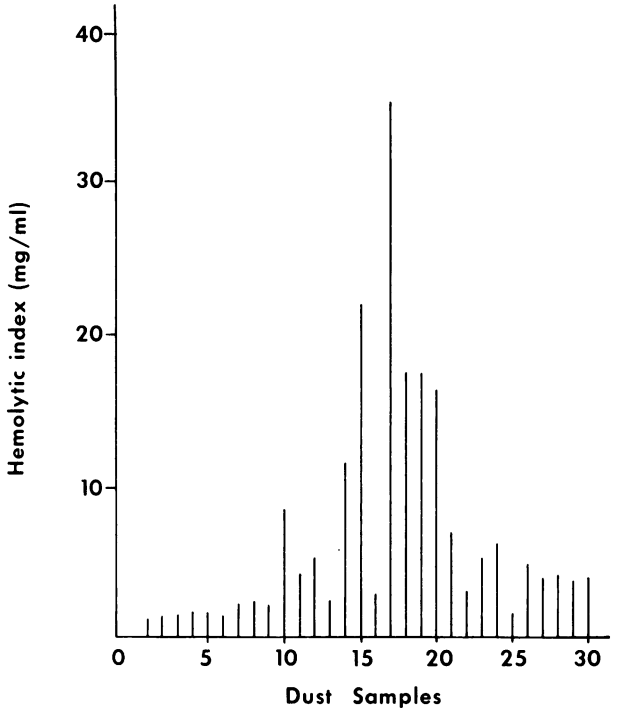

FIGURE 1. Relative hemolytic index (HI) of various dusts: (1) chrysotile, UICC sample (Canadian); (2) chrysotile, Indian (Cuddappa mines); (3) chrysotile, UICC sample (Rhodesian); (4) chrysotile, Indian (Mohanlal Ganj, local asbestos factory); (5) fugitive dust (Mohanlal Ganj at the time of loading); (6) fugitive dust (Mohanlal Ganj, at the time of cutting of asbestos sheets); (7) slate dust, native; (8) slate dust (fugitive) I, sample from Mandsaur slate industry; (9) slate dust (fugitive) II, sample from Mandsaur slate industry; (10) slate dust, Acid-treated; (11) slate dust, base-treated; (12) slate dust, water-treated; (13) slate dust, heat-treated; (14) slate dust, PVP-treated; (15) slate dust, BSA-treated; (16) slate dust, EDTA treated; (17) slate dust, serum treated; (18) mica; (19) hematite; (20) anthophyllite (Indian); (21) tremolite (UICC); (22) anthophyllite (UICC); (23) amosite (UICC); (24) crocidolite (UICC); (25) kaolin; (26) actinolite (UICC); (27) quartz; (28) attapulgite (35% < 5 μ); (29) Attapulgite (< 5 μ). Chrysotile particle size < 30 μ; all the other dusts < 5 μ or otherwise specified. HI = amount of the dust (in mg) needed for 50% hemolysis of 0.2% RBC in 120 min (except chrysotile, where it is 10 min).

le Cancer (UICC) reference sample of chrysotile dust showed the highest hemolytic potential (1.2 mg/mL causing 50% lysis). An Indian variety of chrysotile, collected from Cuddappa District, Andhra Pradesh, India, and some chrysotile used in a local asbestos cement factory (U.P. Asbestos Cement Co., Rai Bareilly, Uttar Pradesh, India), as raw material or collected in the same factory at different places, had a comparable lytic index. Slate dust was also highly lytic. The lytic effect of native kaolin was also as powerful as chrysotile and slate, while quartz was about half as effective. Attapulgite (sample developed as a safe contact insecticide and supplied by Regional Research Laboratory, Hyderabad, India), actinolite and tremolite had membrane toxicity somewhat less than that of quartz. The hemolytic potential (HP = 1/HI) of hematite and the Indian variety of anthophyllite and mica were low.

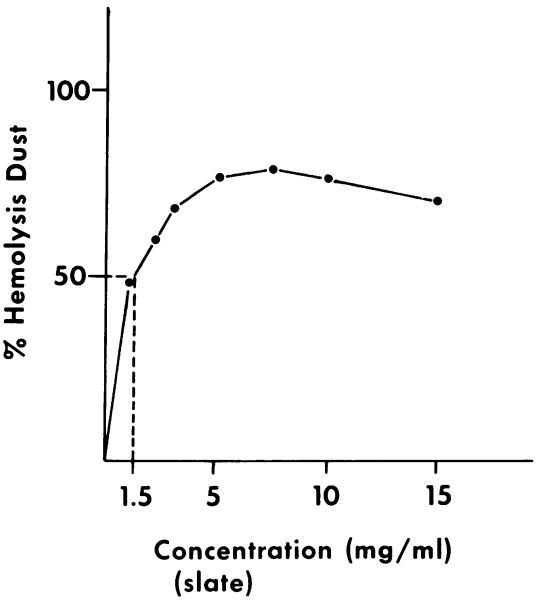

FIGURE 2. Hemolytic dose-response to slate dust. The response is approximately linear up to 4 mg/mL and increases up to 7.5 mg/mL after 2 hr of incubation.

Studies on Slate Dust-Induced Hemolysis

Effect of Dust Concentration on Lytic Activity

In Figure 2, the lytic activity with different native slate dust concentrations is recorded. Hemolytic activity increased up to 7.5 mg; beyond this there was a slow decline. From these data, it follows that 1.5 mg/mL of dust caused 50% lysis, and the lytic index actually was 666 units/g dry dust. At higher concentrations the activity was less, due to increased chances of hemoglobin adsorption.

Effect of Time on Lytic Activity

At all dust concentrations, the rate of lysis was rapid up to 30 min and then slowed until 60 min. Unlike the situation at 5 and 10 mg/mL levels, beyond 60 min hemoglobin release was very much decreased, presumably due to adsorption of hemoglobin on the dust.

The influence of varying both time and concentration is summarized in Figure 3.

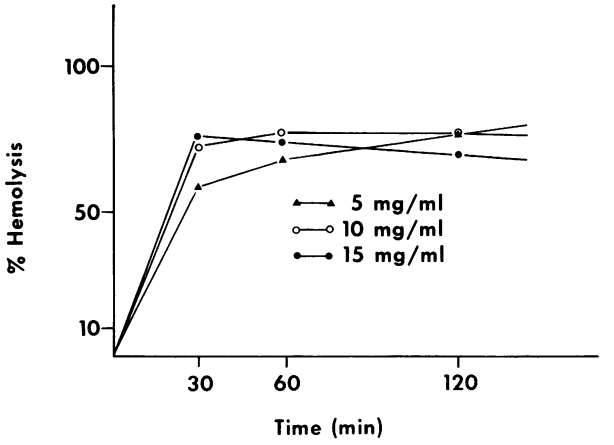

FIGURE 3. Time-dependent hemolytic response to slate dust. At 5 mg/mL the response can be seen to increase up to 120 min. At 10 and 15 mg/mL the response can be seen to plateau in 30 min.

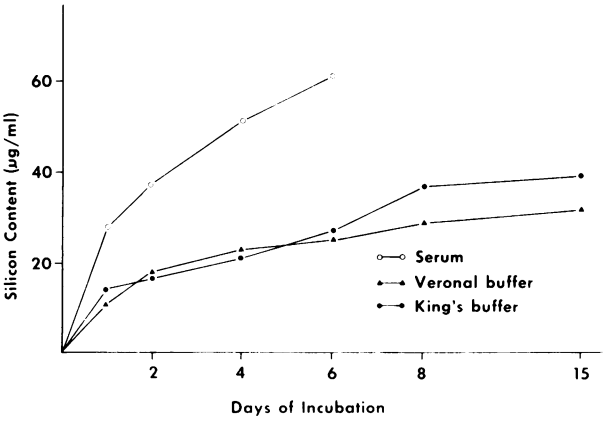

FIGURE 4. Silicon dissolved from slate dust in physiological media. The silicon concentration rose most dramatically in the first incubation day, and was greatest in serum.

Studies on the Solubility of Slate Dust

Dissolution of Silica

In view of the importance of solubility in exerting toxic effect, dissolution of silicic acid in physiological conditions was studied *in vitro* according to a method adopted earlier (*7*). Silica was estimated by the microspectrophotometric adaptation of the method of King et al. (*16*). The solubility of silica eluted from slate dust in serum, King's Ringer buffer, and Veronal buffer at different time intervals is recorded in Figure 4.

In all three media, the dissolution of silicic acid increased with the period of incubation rapidly in the first 24 hr and more slowly later on. Moreover, at all the stages, the solubility in serum was much higher than in the buffers.

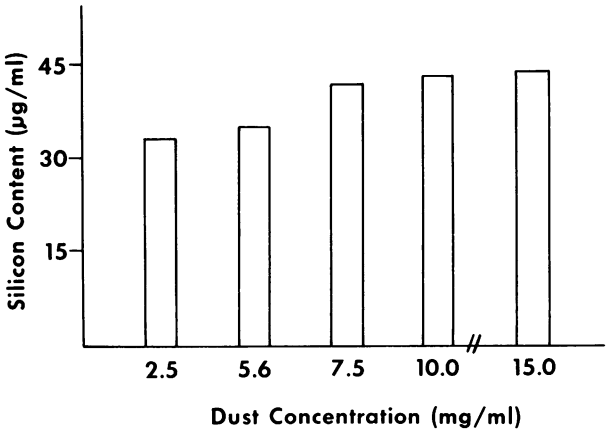

FIGURE 5. Silicon dissolved from slate dust in Ringer's buffer as a function of slate dust concentration. The amount of silicon dissolved plateaus at a concentration of 7.5 mg/mL after 15 days incubation.

The data shown in Figure 5 indicate that the solubility in King's Ringer buffer did not increase in proportion when the concentration of dust was increased. The reason for this could be due to the solution reaching saturation under the experimental conditions or to polymerization of the silicic acid. At pH values above 7.0, the chances of hydroxide formation of the cations on the dust surface to reduce solubility and at higher dust surface/solvent ratios, this possibility increases.

Dissolution of Magnesium

The ability of three solvents to elute magnesium (Mg) from dust, as determined by atomic absorption spectrophotometry, is represented in Figure 6. Dissolution of Mg in King's Ringer and Veronal buffer was low but increased gradually as the period of incubation was extended. With serum, the rate of dissolution was much more rapid, and after 6 days, the Mg dissolved in serum was almost 4-fold that dissolved in buffer.

Adsorption of Serum Protein on Dusts

During the solubility studies in serum, it was found that at 1 and 2 days post-incubation, 20 mg of dust adsorbed 6.8 and 26.4 mg proteins from 4.0 mL serum. At 4 days, the adsorption was similar to that at 2 days, indicating saturation of sites on dust surface. Protein measurement was performed by the method of Lowry et al. (*17*).

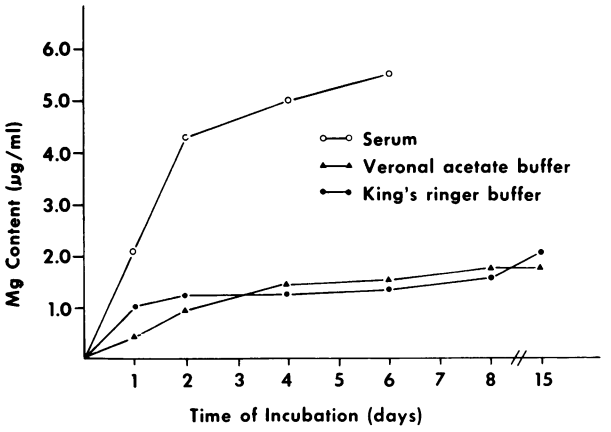

FIGURE 6. Magnesium dissolved from slate dust in physiological media. The dissolution in serum can be seen to be much greater than that in the buffers tested.

Effect of Coating of Dust Surface on Hemolysis

A 50 mg portion of dust was separately preincubated with 2 mg of bovine serum albumin, 2 mL EDTA ($M \times 10^{-3}$, pH 7.4) and 2 mL serum for 3 hr at 37°C and with various lipids (35.5 mg of each lipid) at 37°C for 24 hr with constant shaking. The dust was recovered by centrifugation, and 0.2% suspension of erythrocytes was added.

From the data recorded in Table 1, it is seen that plasma and albumin reduced the hemolytic ability of native dust by 88 and 83%, respectively. Under physiological conditions, coating with PVP was also effective in reducing the hemolytic activity about 70%. Lecithin and sphingomyelin reduced slate dust-induced hemolysis 60 and 50%, respectively. These treatments reduce hemolysis only, but did not affect the time course of reaction. Pretreatment with EDTA did not cause any effect on lysis.

Effect of Acid, Alkali and Heat Treatment

The hemolytic effect of pretreatment of 100 mg dust with 5.0 mL 0.1 *N* HCl or with 0.1 *N* NaOH at 37°C for 24 hr, followed by exhaustive washing and drying on its lytic property, is summarized in Table 2. Data for dust heated at 600°C for 2 hr and exhaustively washed with water are also given for comparative purposes.

Heat treatment did not affect the hemolysis; the values were more or less similar to that of native dust. Exhaustive washing with water reduced hemolysis, apparently due to the removal of active components by solubilization, thereby altering surface structure and charge. Alkali treatment caused reduction in hemolytic ability, even though it was

Table 1. Effect of coating of dust surface on hemolysis.

No.	Treatment	Hemolysis, %
1	Normal dust	72.8
2	PVP-treated dust	22.8
3	BSA-treated dust	11.4
4	EDTA-treated dust	72.8
5	Serum treated	7.1
6	Sialic acid	71.8
7	Cholesterol	70.1
8	Choline chloride	70.8
9	Lecithin	28.5
10	Sphingomyelin	38.5

Table 2. Effect of acid, alkali, water and heat treatment on hemolysis.

No.	Treatment	Hemolysis, %
1	Normal slate dust	70.2
2	Acid-treated	30.7
3	Alkali-treated	58.9
4	Water-treated	47.4
5	Heat-treated	69.2

less effective than exposure to water. Alkali treatment may cause the formation of a protective metallic hydroxide layer on dusts reducing rate of dissolution (*17*). This view is supported by the observation that, whereas alkali treatment caused only removal of 3.3 µg Si/mL, water extracted 36.4 µg/mL of Si. Acid treatment led to 113.6 µg/mL loss of Si from 100 mg of dust in 50 mL medium. Simultaneously, the hemolytic effect was also markedly reduced by acid treatment to less than 50% that of the untreated form. Acid treatment could favor Si dissolution by removal of cations. Thus, alterations in the physicochemical properties, especially Si content and the structure, could reduce the lytic effect.

Erythrocyte Ghost Membrane

To elucidate the membrane, changes may be responsible for hemolysis. Erythrocyte ghost membrane was prepared according to Dodge et al. (*18*). The chemical composition of ghost cells prepared by hypotonic shock alone and after dust treatment indicated significant differences in protein phospholipids, sialic acid, and glucosamine contents (Table 3) showing changes in the membrane with dust treatment.

Discussion

The various particulate pollutants screened for membrane toxicity differed markedly in their hemolytic potential, indicating probable variations in

Table 3. *In vitro* studies on ghost membrane.

	Protein mg/mL	Silica content μg/mg protein		Carbohydrate content μg/mg protein			mg/mL	Total phospholipid estimated as P_i liberated
		TCA sup	TCA ppt	Glucos-amine	Sialic acid	Glucuronic acid		mg/mg protein
Control	6.4	0.82	0.0	0.0143	0.0096	0.0075	3.0	0.468
Experimental	5.09	2.5	5.2	0.0054	0.0182	0.0072	1.45	0.284

relative toxicity. Chrysotile was among the most lytic, the Indian sample being as effective as its international counterparts (*19, 20*). Since the samples collected from a local asbestos factory also showed similar potency as the native dust, the changes of occupational hazard on prolonged inhalation are indicated. The hemolytic potentials for other dusts were generally of a lower magnitude. Slate mine dusts from Mandsaur showed very powerful hemolytic activity and, therefore, may be potentially toxic.

Even though the hemolytic potential of slate dust was comparable to that of chrysotile, the pattern of lysis seemed to differ in a few respects. Slate-induced lysis, unlike chrysotile, was unaffected by EDTA and heat treatment, but partially reduced by PVP. Apparently, these treatments affect both dusts differently due to differences in chemical and physical properties. The target molecules involved in membrane damage may be different. Since coating of slate dust with serum or albumin caused a gradual decrease in the lysis, chemical interaction with the dust surface may be involved. The adsorption of protein on slate dust also supports this. Further, this observation raises the possibility as to what protein-coated dust, similar to asbestos bodies, could be detected in the sputum of slate workers.

Dissolution of silica in human serum from slate dust was of high magnitude. Earlier studies with various silicate dust showed that solubility is one of the factors involved in toxic effects, especially *in vitro* hemolysis and cytotoxicity (*9*). As with these silicates (*6*), treatment of the slate dust altered both solubility and lytic potential in a parallel manner. Thus, silicic acid could be a factor involved in the lysis by slate, as in the case of quartz (*21*).

Because of surface change in the ghost membrane, it seems that carbohydrate moieties are the sites of action in the increased hemolysis with slate dust in agreement with the views of Harington (*20*).

It may be concluded that the interaction of soluble dust constituents, rather than the fiber itself, with erythrocyte membrane, its carbohydrate components and specific proteins, deserves more detailed study for the elucidation of the molecular mechanism of toxicity of particulate air pollutants.

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